Virology 435 (2013) 385–394

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

DDB1 is a cellular substrate of NS3/4A protease and required for hepatitis C virus replication

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ARTICLE INFO

Article history:
Received 15 July 2012
Returned to author for revisions
11 October 2012
Accepted 15 October 2012
Available online 5 November 2012

Keywords:
HCV
NS3/4A
DDB1
Replication

ABSTRACT

Hepatitis C virus (HCV) infection often causes long-term persistent hepatitis, which eventually leads to liver cirrhosis and hepatocellular carcinoma. HCV-encoded NS3/4A protease plays an important role in HCV immune evasion by cleaving key adapter proteins VISA and TRIF of the RIG-I-like receptors and Toll-like receptors mediated interferon (IFN) induction pathways. To further understand the roles of NS3/4A in HCV life cycle, we identified DDB1 as a cellular substrate of NS3/4A protease by biochemical purification and mass spectrometry analysis. NS3/4A interacted with DDB1 and cleaved DDB1 in HCV-infected cells. Mutagenesis indicated that NS3/4A cleaved DDB1 at the residue of C378. Overexpression of DDB1 potentiated HCV replication, whereas knockdown of DDB1 dramatically inhibited HCV replication. Furthermore, our data indicated that the cleavage of DDB1 by NS3/4A protease was required for HCV replication. Our findings suggest that DDB1 is a cellular substrate of NS3/4A required for HCV replication and provide new insight into the interaction between HCV and host cells.

Introduction

Hepatitis C Virus (HCV) infection is a global health threat. More than 170 million people are infected by HCV worldwide, and many of them are subjected to chronic infection and high risk of developing liver cirrhosis and hepatocellular carcinoma (Lauer and Walker, 2001). HCV is a positive-strand RNA virus that belongs to the *Flaviviridae* family. The HCV genome encodes a single polyprotein of ~3000 amino acids, which is processed by a combination of host and viral proteases into at least 10 structural and nonstructural proteins. The structural proteins include core, E1 and E2, which are important for assembly of viral particles. The non-structural proteins include p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B, which are important for HCV replication and interaction with the host (Bartenschlager et al., 2011).

The HCV NS3/4A protease consists of two subunits, NS3 and NS4A. NS3 contains an N-terminal serine protease domain and a C-terminal RNA helicase domain. NS4A acts as a cofactor, which tethers the holoenzyme complex to an intracellular membrane compartment. Besides playing an essential role in processing viral proteins, the NS3/4A protease also cleaves cellular proteins. Recent studies have demonstrated that NS3/4A can cleave VISA (also known as IPS-1, MAVS, Cardif) (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005) at C508 and TRIF at C372, leading to blockade of the RIG-I-like (RLR)- and Toll-like receptor (TLR)-mediated induction of type I interferons (IFNs) and evasion of the innate antiviral response (Cheng et al., 2006; Li et al., 2005a; Li et al., 2005b; Meylan et al., 2005). It has also been reported that NS3/4A is involved in activation of EGF-induced signaling by cleaving T-cell protein tyrosine phosphatase (TC-PTP) (Brenndorfer et al., 2009). However, whether NS3/4A is involved in other virus-host interactions through targeting unidentified cellular substrates is largely unknown.

In this report, we identified DDB1, a core subunit of the Cul4 -based ubiquitin ligase complex (Chen et al., 2001), as a bona fide cellular substrate of NS3/4A protease. Our findings suggest that DDB1 is required for HCV replication and provide new insight into the interaction between HCV and the host cells.

Results

Identification of DDB1 as an NS3/4A-associated protein

NS3/4A protease plays a vital role in HCV life cycle and virus-cell interactions. To identify potential new cellular substrates of NS3/4A, we performed biochemical purification experiments. An expression plasmid for N-terminal Flag epitope-tagged NS3/4A was transfected into 293 cells. NS3/4A-associated proteins were purified by anti-Flag antibody affinity purification and

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http://dx.doi.org/10.1016/j.virol.2012.10.025

identified by tandem mass spectrometry analysis. By comparing with proteins identified from control samples, we identified DDB1 as a candidate protein specifically associated with NS3/4A (Supplementary Fig. 1). DDB1, a 127 kDa protein containing three β-propeller domains (BPA–BPC) (Li et al., 2006), is a core subunit of the Cul4 -based ubiquitin ligase complex. Conserved from yeast to human, the Cul4-DDB1 ubiquitin ligase complex regulates diverse cellular functions such as DNA repair (Chen et al., 2001; Kapetanaki et al., 2006; Sugasawa et al., 2005; Wang et al., 2006), DNA replication (Higa et al., 2003; Hu et al., 2004) and transcription (Wertz et al., 2004), and can also be subverted by pathogenic viruses to benefit viral infection (Andrejeva et al., 2002a; Bergamaschi et al., 2009; Casey et al., 2010; Didcock et al., 1999; Li et al., 2006; Li et al., 2010; Schrofelbauer et al., 2007; Sharova et al., 2008).

To determine whether NS3/4A indeed interacts with DDB1, we performed transient transfection and coimmunoprecipitation experiments. The results indicated that NS3/4A could interact with DDB1 (Fig. 1A). In these experiments, NS3/4A did not interact with Cul4A and DDB2, suggesting that NS3/4A interacts with DDB1 specifically. However, NS3/4A could impair the interaction between DDB1 and Cul4A (Supplementary Fig. 2). Furthermore, endogenous coimmunoprecipitation experiments indicated that DDB1 interacted with NS3/4A in hepatic Huh-7 cells infected with the HCV genotype 2a clone Japanese Fulminant Hepatitis 1 (JFH-1) (Fig. 1B). Interestingly, we also found that DDB1 migrated slower in the presence of NS3/4A (Fig. 1A), and HCV infection also caused slower migration of cellular DDB1 (Fig. 1B). The simplest explanation for these observations is that NS3/4A may directly or indirectly mediate post-translational modification(s) of DDB1.

DDB1 is a substrate of NS3/4A protease

NS3/4A is a serine protease which can cleave its substrates. In our coimmunoprecipitation experiments, we found that DDB1 was cleaved when co-transfected with NS3/4A in 293 cells (Fig. 1A). In Huh-7 cells, endogenous DDB1 was also cleaved after JFH-1 infection (Fig. 1B). These results suggest that DDB1 is a bona fide cellular substrate of NS3/4A. To confirm that DDB1 is cleaved by NS3/4A protease activity, we constructed a N-terminal and a C-terminal Flag-tagged DDB1 expression plasmid respectively. We found that NS3/4A cleavage resulted in a ~45 kDa N-terminal fragment and a ~82 kDa C-terminal fragment (Fig. 2A). The added molecular size of these two fragments is consistent with the expected size of 127 kDa of the full-length DDB1. The cleavage of DDB1 by NS3/4A was inhibited by the NS3/4A inhibitor VX-950 (Fig. 2A). We also made recombinant DDB1 by an in vitro transcription/translation method and showed that NS3/4A could cleave recombinant DDB1 (Supplementary Fig. 3). In this experiment, an enzymatic-inactive mutant, NS3/4A (S139A), did not cleave DDB1 (Supplementary Fig. 3). These results confirmed that DDB1 is cleaved by NS3/4A protease.

NS3/4A cleaves DDB1 at C378

It has been shown that NS3/4A cleaves at the C-terminus of a C or T residue within a loosely defined consensus sequence (E/D)*xxxx*(C/T)(S/A) (where *x* denotes any amino acid), which can be found at the junction of HCV non-structural proteins (Fig. 2B) (Grakoui et al., 1993). Amino acid sequence analysis of human DDB1 showed that $_{303}$ELLGE**TS**$_{309}$ is a potential NS3/4A recognition sequence, with T308 serves as the potential cleavage site. However, cleavage at T308 is predicted to generate an N-terminal fragment of ~35 kDa, which is inconsistent with the actually cleaved N-terminal fragment of ~45 kDa (Figs. 1 and 2A). Mutation of T308 to alanine did not affect its cleavage by NS3/4A (Supplementary Fig. 4A), also suggesting that this residue is not a NS3/4A cleavage site.

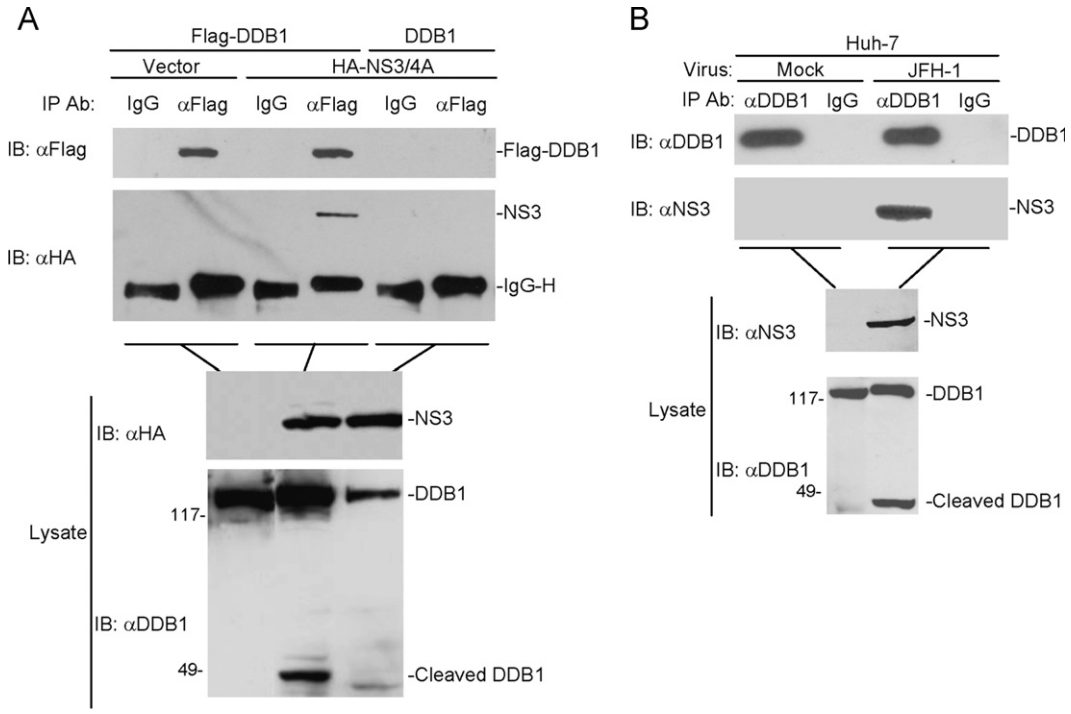

Fig. 1. DDB1 interacts with and cleaved by NS3/4A. (A) DDB1 interacts with NS3/4A in overexpression system. The 293 cells were transfected with the indicated plasmids. Coimmunoprecipitation was performed with anti-Flag or control IgG. The immunoprecipitates were analyzed by immunoblot with anti-Flag anti-HA. The lysates were analyzed by immunoblots with anti-DDB1 or anti-HA. (B) Endogenous DDB1 interacts with NS3/4A in JFH-1 infected cells. Huh-7 cells (5×10^7) were mock-infected or infected with JFH-1 (Multiplicity of Infection, MOI: 0.3) for 3 days. Coimmunoprecipitation was performed with anti-DDB1 or control IgG. The immunoprecipitates were analyzed by immunoblot with anti-DDB1 and anti-NS3. The lysates were analyzed by immunoblots with anti-DDB1 or anti-NS3.

In searching for other NS3/4A targeting sequences in DDB1, we found another sequence, $_{373}$GQLVT**C**S$_{379}$ in DDB1. This was primarily based on two previous observations: (1) significant alteration of cleavage efficiency is not found when E or D at position P6 of the NS3/4A targeting sequence is replaced by G, F or N (Komoda et al., 1994), and (2) NS3/4A prefers C to T at P1 position and the P6 position of the TRIF protein is P rather than E or D (Li et al., 2005a) (Fig. 2B). A mutant of DDB1 in which C378 was changed to arginine was completely resistant to NS3/4A cleavage (Fig. 2C), suggesting that NS3/4A cleaves DDB1 at C378. Consistently, the cleaved N-terminal fragment of DDB1 was equal in size with ectopically expressed DDB1(1–378) (Fig. 2D). To confirm that C378 is the cleavage site of DDB1, we further mutated two conserved amino acids in the cleavage motif individually, including aa379 from S to N and aa373 from G to A. We found that both DDB1(S379N) and DDB1(G373A) were resistant to cleavage by NS3/4A (Supplementary Fig. 4B). In coimmunoprecipitation experiments, DDB1(C378R) could still interact with NS3/4A (Supplementary Fig. 5), excluding the possibility that mutation of C378 to arginine disrupts the general structure of DDB1. Taken together, our results suggested that DDB1 is cleaved after C378 by NS3/4A.

Since DDB1 is cleaved by NS3/4A, we wondered whether HCV infection affects the cellular localization of DDB1. Confocal microscopy with GFP-tagged DDB1 indicated that DDB1 was mostly localized in the cytoplasmi, and this localization was not affected by JFH-1 infection (Supplementary Fig. 6).

DDB1 plays a critical role in HCV replication

After identifying DDB1as a cellular substrate of NS3/4A protease, we investigated the roles of DDB1 in HCV replication. Firstly, we overexpressed DDB1 in Huh-7 cells and then examined its effect on replication of JFH-1 virus by RT-qPCR analysis of viral RNA level. As shown in Fig. 3A, overexpression of DDB1 markedly up-regulated replicated viral RNA level after JFH-1 virus infection in a dose-dependent manner. We then determined the effect of DDB1 deficiency on JFH-1 virus replication. We established two-independent DDB1-RNAi knockdown Huh-7 cell lines by targeting different sites of DDB1 mRNA. The expression levels of DDB1 in these two cell lines were dramatically down-regulated in comparison to a control cell line (Fig. 3B). The DDB1-RNAi knockdown cells were infected with JFH-1 virus for 3 days and then viral RNA levels were examined by RT-qPCR . As shown in Fig. 3B, knockdown of DDB1 dramatically inhibited viral RNA level after JFH-1 infection. In addition, immunofluorescent staining experiments with an antibody against the JFH-1 envelope E2 protein revealed that the intracellular E2 levels in DDB1-knockdown cells were much lower than that in control cells (Fig. 3C). We also collected supernatants from JFH-1-infected DDB1-RNAi knockdown or control cells and performed foci formation experiments with naïve Huh-7 .5.1 cells (Zhong et al., 2005). The results indicated that knockdown of DDB1 dramatically reduced the level of newly secreted viruses to ~0.1% of the control sample (Fig. 3D). Although we have used two-independent DDB1-RNAi knockdown

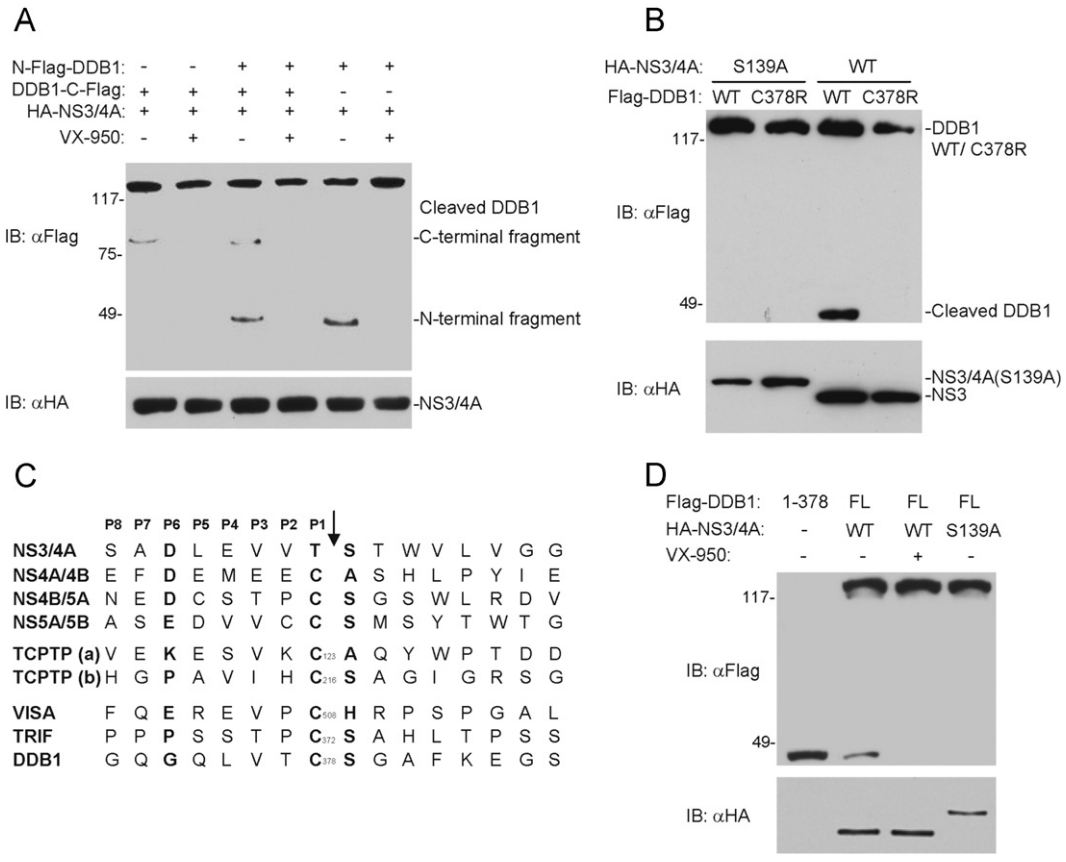

Fig. 2. NS3/4A cleaves DDB1 at C378. (A) Cleavage of DDB1 by NS3/4A is inhibited by the NS3/4A inhibitor VX-950. The 293 cells were transfected with N-terminal or C-terminal Flag-tagged DDB1 (N-Flag-DDB1 or DDB1-C-Flag respectively) and HA-NS3/4A. The transfected cells were treated with VX-950 (0.2 mM) or left untreated for 1 day before immunoblot analysis with anti-Flag or anti-HA. (B) Alignment of the junction sequences of NS proteins of HCV and the potential NS3/4A cleavage sites in TC-PTP, VISA, TRIF and DDB1. (C) NS3/4A cleaves DDB1 at C378. The 293 cells were transfected with the indicated plasmids and cells lysates were analyzed by immunoblots with anti-Flag or anti-HA. (D) DDB1 N-terminal cleavage product migrated similarly to overexpressed DDB1(1–378) mutant. The 293 cells were transfected with the indicated plasmids, treated with VX-950 or left untreated for 1 day before immunoblot analysis with with anti-Flag or anti-HA.

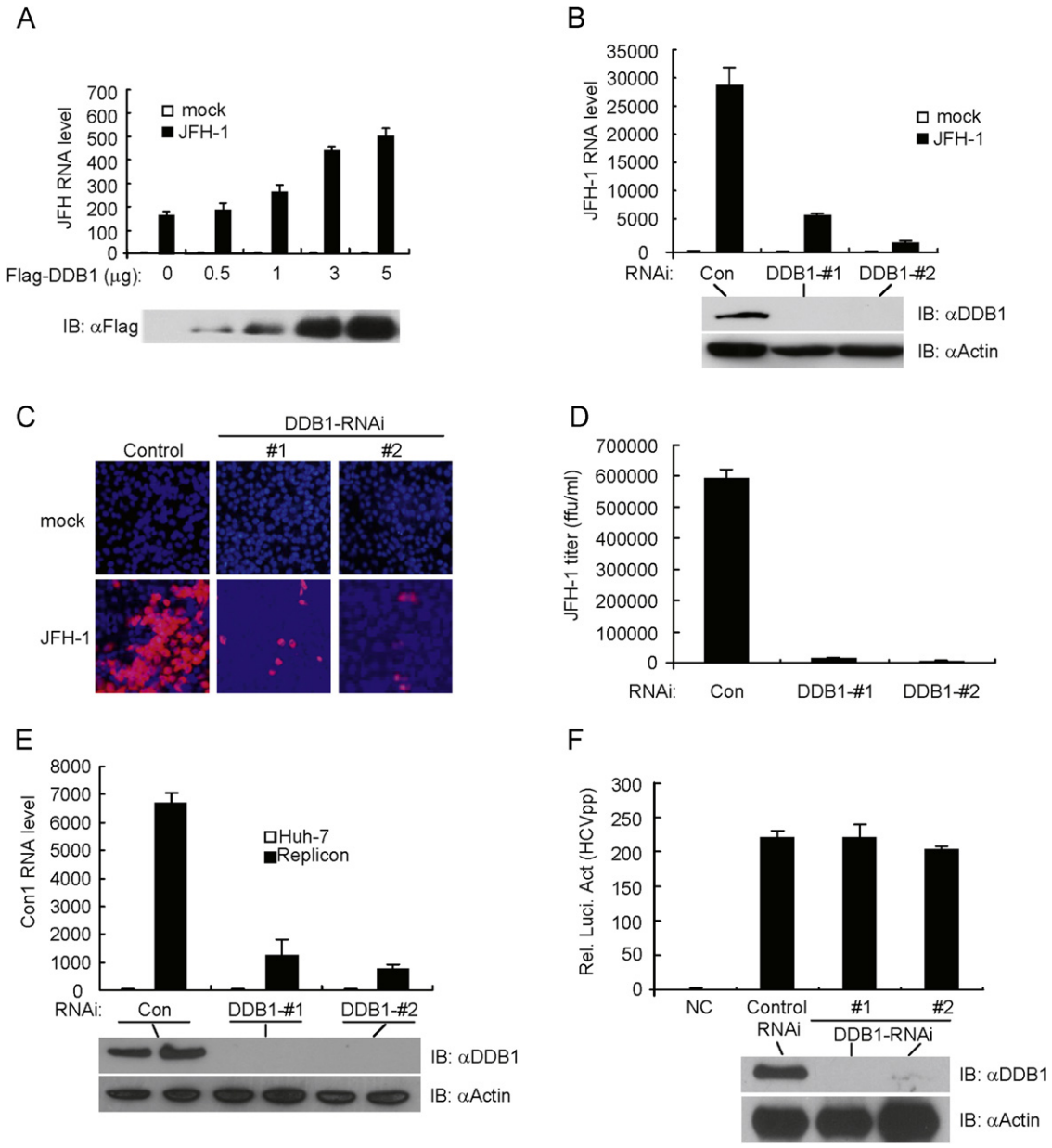

Fig. 3. DDB1 plays a critical role in HCV replication. (A) Overexpression of DDB1 potentiates HCV RNA replication. Huh-7 cells (1×10^6) were transfected with the indicated amounts of Flag-DDB1 plasmid for 24 h and then cells were split and mock-infected or infected with JFH-1 (MOI: 0.3) for 3 days. Intracellular HCV RNA levels were determined by RT-qPCR and normalized to cellular GAPDH mRNA levels. The uninfected cell lysates were analyzed by immunoblots with anti-Flag or anti-β-actin. Graphs show mean $\pm$ SD, $n=3$. (B) Knockdown of DDB1 inhibits HCV RNA replication. Control or DDB1-RNAi knockdown Huh-7 cells were mock-infected or infected with JFH-1 (MOI: 0.3) for 3 days. Intracellular HCV RNA levels were then determined by RT-qPCR and normalized to GAPDH mRNA levels. The uninfected cells lysates were also analyzed by immunoblots with anti-DDB1 or anti-β-actin. Graphs show mean$+$SD, $n=3$. (C) Knockdown of DDB1 inhibits HCV protein expression. Control or DDB1-RNAi knockdown Huh-7 cells were mock-infected or infected with JFH-1 for 3 days, and the cells were then analyzed by immunofluorescent staining with anti-E2 (red), and Hoechst (blue). (D) Knockdown of DDB1 inhibits production of infectious HCV particles. Control or DDB1-RNAi knockdown Huh-7 cells were mock-infected or infected with JFH-1 for 24 h. The cells were completely washed and fresh complete medium was added for 48 h. The JFH-1 infected medium was collected and diluted for infection of Huh-7.5.1 cells. Three days later, cells were analyzed by immunofluorescent staining with anti-E2 and HCV titers were calculated by counting positive stained cells foci. Graphs show mean $\pm$ SD, $n=3$. (E) Knockdown of DDB1 inhibits RNA replication of HCV subgenomic replicon. Control or DDB1-RNAi knockdown Huh-7 cells and Huh-7 Con1 subgenomic replicon cells were cultured for 3 days. The cells (2×10^6) were collected and intracellular HCV RNA levels were determined by RT-qPCR and normalized to cellular GAPDH mRNA levels. Cell lysates were analyzed by immunoblots with anti-DDB1 or anti-β-actin. Graphs show mean $\pm$ SD, $n=3$. (F) DDB1 has no effects on HCV entry. Control or DDB1-RNAi knockdown Huh-7 cells were infected with HCVpp for 3 days (NC: Negative Control, HCVpp pakaging without HCV E1E2). The lysates of infected cells were assayed by luciferase reporter assays and immunoblots with anti-DDB1 or anti-β-actin. Graphs show mean $\pm$ SD, $n=3$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cell lines for above experiments and obtained similar results, we further confirmed the conclusion by reconstitution experiments with RNAi off-target nonsense mutants of DDB1. Rescue of DDB1 expression by these mutants in DDB1-RNAi knockdown cells markedly restored JFH-1 RNA replication and viral particle production (Supplementary Fig. 7). Moreover, we found that knockdown of DDB1 dramatically suppressed the Con1 RNA level in a Con1 sub-genomic HCV replicon cells (29) (Fig. 3E). To exclude the possibility that DDB1 affects HCV entry to host cells, we utilized HCVpp for our analysis. HCVpp is a pseudotyped retroviral particle which can be produced by co-transfection of a plasmid containing envelope-deficient HIV genome pNL4-3 .Luc.R-.E- and a plasmid expressing HCV glycoproteins in 293 cells. Since HCVpp only contains the E1 and E2 envelope proteins

of HCV, it is incapable of replicating in cells. However HCVpp can be used to monitor whether HCV enters host cells through quantitative luciferase reporter assays. Reporter assays performed after HCVpp infection indicated that knockdown of DDB1 had no significant effect on HCV entry (Fig. 3F). Taken together, these results suggest that DDB1 plays a critical role in HCV replication.

DDB1 cleavage is required for HCV replication

Since DDB1 is cleaved by NS3/4A and required for HCV replication, we next investigated the effects of DDB1 cleavage on HCV replication. We established stable Huh-7 cell lines expressing wild-type and the C378R, S379N and G373A mutant DDB1. These cells were mock infected or infected by JFH-1 virus for 3 days, and then the intracellular viral RNA levels, E2 expression and secretion of viral particles were measured. The results indicated that JFH-1 RNA replication and viral particle production in the C378R, S379N and G373A mutant expressing cells were dramatically suppressed in comparison with control cells (Fig. 4 A–C and Supplementary Fig. 8). Since expression of endogenous DDB1 exists in these stable cell lines, we established stable cell lines for RNAi off-target mutants of wild-type DDB1 and DDB1(C378R) in DDB1-RNAi knockdown cells, and performed JFH-1 infection experiments with these cells. Again, we found that JFH-1 RNA levels were markedly reduced in DDB1(C378R) cells (Fig. 4D). Taken together, these results suggest that DDB1 cleavage is required for HCV repplication.

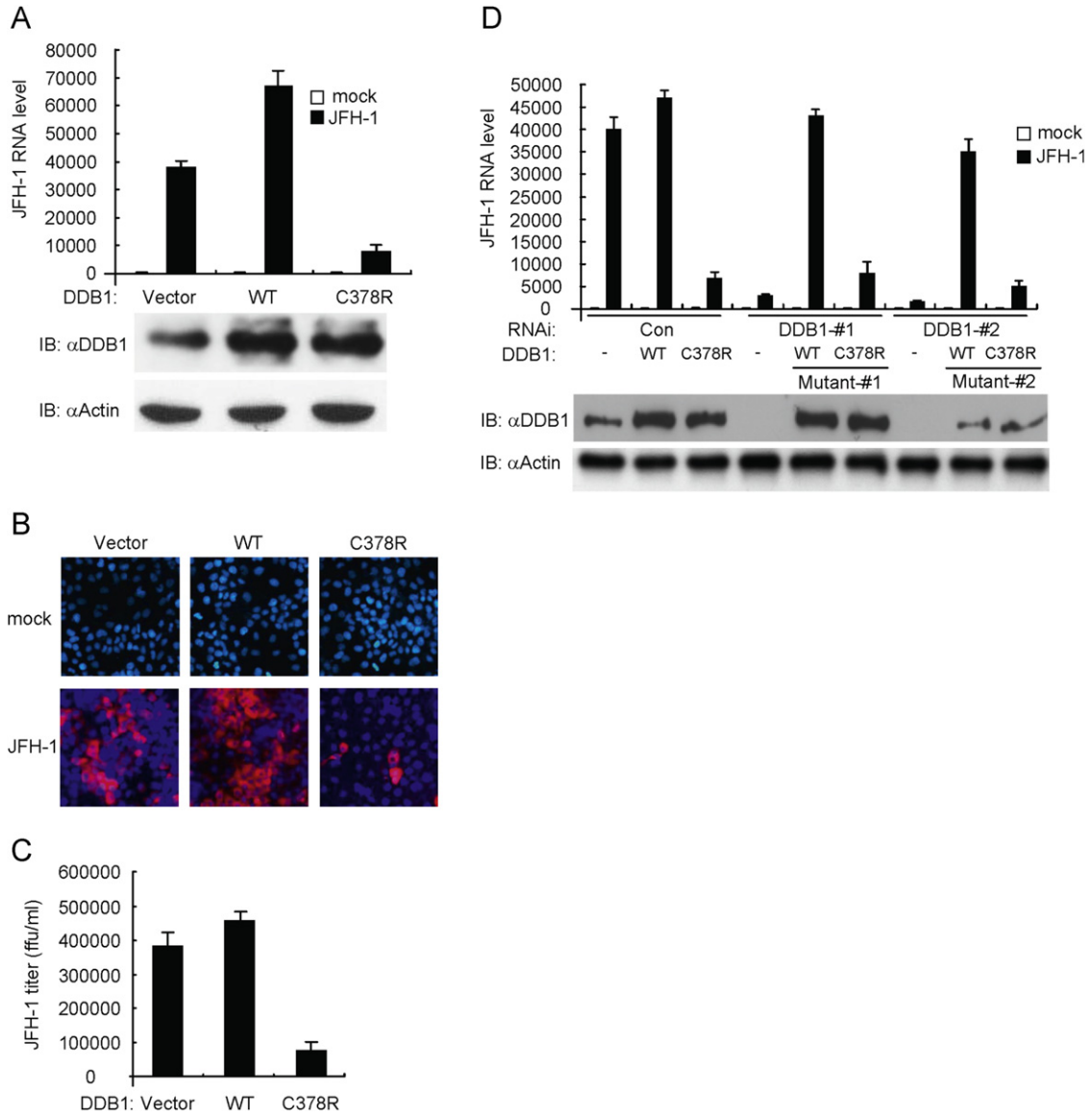

Fig. 4. DDB1 cleavage is required for HCV replication. (A) The indicated stable cell lines were mock-infected or infected with JFH-1 (MOI: 0.3) for 3 days. Intracellular HCV RNA levels were then determined by RT-qPCR and normalized to GAPDH mRNA levels. The uninfected cells lysates were also analyzed by immunoblots with anti-DDB1 or anti-β-actin. Graphs show mean+SD, $n=3$. (B) The indicated stable cells were mock-infected or infected with JFH-1 (MOI: 0.3) for 3 days. The cells were then analyzed by immunofluorescent staining with anti-E2 (red), and Hoechst (blue). (C) The indicated stable cell lines were mock-infected or infected with JFH-1 (MOI: 0.3) for 24 h. The cells were completely washed and fresh medium was added for 48 h. The JFH-1-containing medium was collected and diluted for infection of Huh-7.5.1 cells. Three days later, cells were analyzed by immunofluorescent staining with anti-E2 and HCV titers were calculated by counting positive stained cells foci. Graphs show mean+SD, $n=3$. (D) Control or DDB1-RNAi knockdown Huh-7 cells were stably transduced with empty vector, DDB1, DDB1(C378R), off-target nonsense mutants of DDB1 or DDB1(C378R) respectively. Two days later, cells were mock-infected or infected with JFH-1 (MOI: 0.3) for 3 days. Intracellular HCV RNA levels were then determined by RT-qPCR and normalized to GAPDH mRNA levels. The cells lysates were also analyzed by immunoblots with anti-DDB1 or anti-actin. Graphs show mean+SD, $n=3$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

DDB1 cleavage products do not affect the HCV infection

Our foregoing results suggest that DDB1 cleavage is required for HCV replication. To investigate the significance of DDB1 cleavage products on HCV infection, we established stable cell lines expressing DDB(1–378), DDB1(379–1140), or both fragments together by retroviral-mediated gene transfer. These cell lines were mock infected or infected with JFH-1 virus for 3 days and then intracellular HCV RNA, viral-particles in the cytoplasm and culture medium were analyzed. The results indicated that overexprssion of the cleaved fragments alone or together had no marked effects on HCV replication (Fig. 5A–C). To eliminate the possibility that endogenous DDB1 may interfere the results in these experiments, DDB1-RNAi knockdown cells were transduced

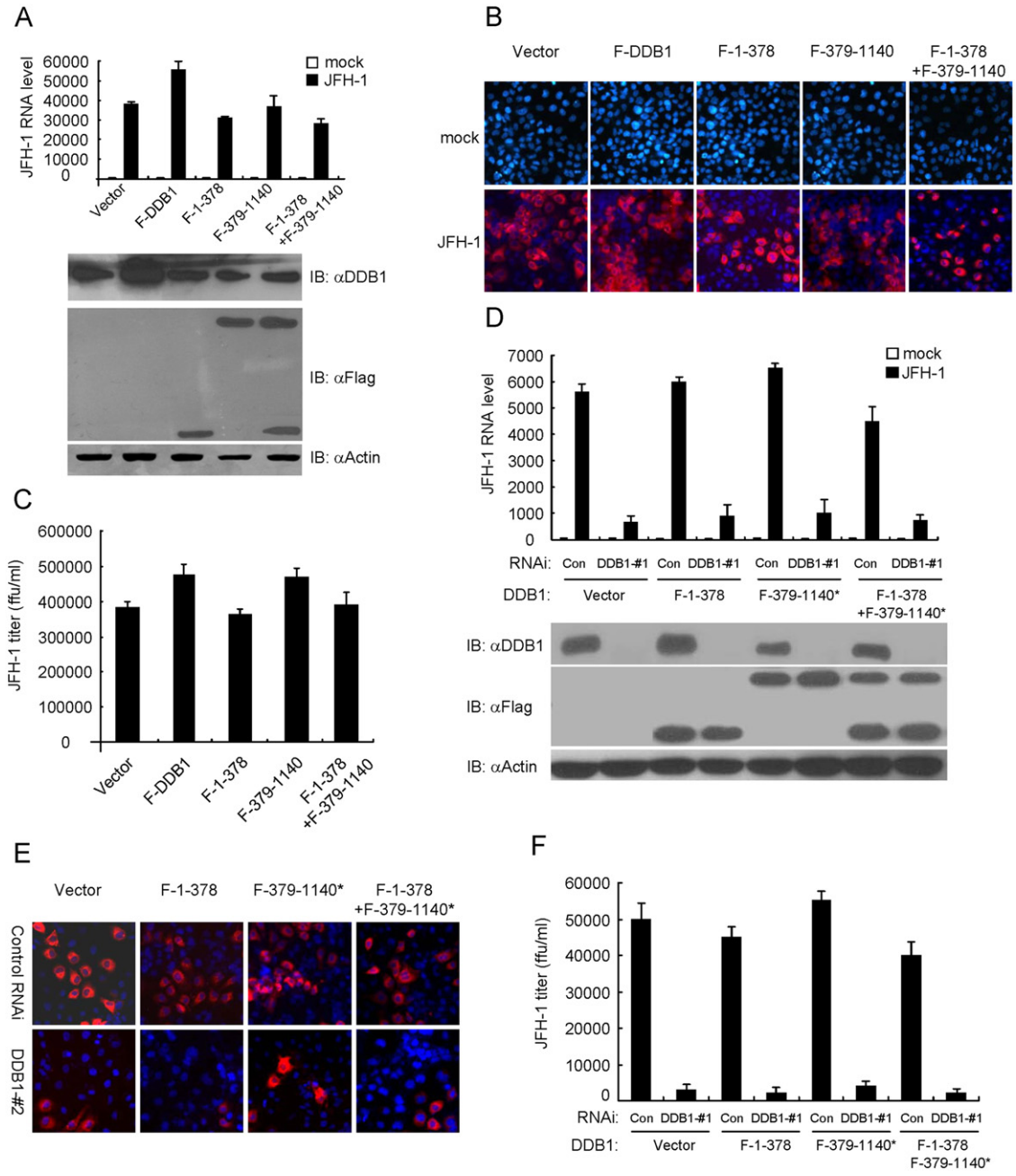

Fig. 5. DDB1 cleavage products do not affect the HCV infection. (A) Huh-7 cells stably transduced with the indicated DDB1 truncation mutants were mock-infected or infected with JFH-1 (MOI: 0.3) for 3 days. Intracellular HCV RNA levels were then determined by RT-qPCR and normalized to GAPDH mRNA levels. The uninfected cell lysates were analyzed by immunoblots with anti-DDB1 or anti-β-actin. Graphs show mean+SD, $n=3$. (B) Huh-7 cells stably transduced with the indicated DDB1 truncation mutants were mock-infected or infected with JFH-1 (MOI: 0.3) for 3 days. The cells were then analyzed by immunofluorescent staining with anti-E2 (red), and Hoechst (blue). (C) Huh-7 cells stably transduced with the indicated DDB1 truncation mutants were mock-infected or infected with JFH-1 (MOI: 0.3) for 24 h. The cells were completely washed and fresh complete medium was added for 48 h. The JFH-1 infected medium was collected and diluted for infection of Huh-7.5.1 cells. Three days later, cells were analyzed by immunofluorescent staining with anti-E2 and HCV titers were calculated by counting positive stained cells foci. Graphs show mean+SD, $n=3$. (D–F) Control or DDB1-RNAi-#1 (targeted sequence is within the cDNA fragment encoding aa379–1140) transduced Huh-7 cells were further transfected with empty vector, Flag-DDB1(1–378), Flag-DDB1(379–1140*) (*, a RNAi off-target mutant),or a combination of Flag-DDB1(1–378) and Flag-DDB1(379–1140*) by Lipofectamine 2000. One day post transfection, the cells were split and mock infected or infected with JFH-1(MOI: 0.3) for 3 (D, E) or 1 (F) day. Intracellular HCV RNA levels (D), intracellular viral particles (E), or viral titers in the medium (F) were then determined as described in (A–C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with the DDB1 truncations harboring RNAi off-target mutations, infected with JFH-1 and then similar analyses were performed. Again, the results indicated that overexpression of DDB1 cleavage fragments had no marked effects on HCV replication (Fig. 5D–F).

Discussion

NS3/4A protease is not only essential for HCV polyprotein processing, it is also a critical player in viral subverting and evading of host immune responses (Bartenschlager et al., 1993; Lei et al., 2009; Loo et al., 2006). Despite its key roles in HCV-host interaction, few cellular substrates of NS3/4A have been identified. Previous studies have demonstrated that TRIF and VISA are NS3/4A substrate and cleavage of these proteins leads to suppression of the TLR- and RLR-mediated innate antiviral responses (Li et al., 2005a; Li et al., 2005b). It has also been shown that cleavage of TC-PTP by NS3/4A is involved in activation of the EGF-induced signaling pathways (Brenndorfer et al., 2009). Recently, a protein called YB-1 was reported to be a NS3/4A-interacting protein which can affect HCV replication and viral-particle production (Chatel-Chaix et al., 2011). In this report, we identified DDB1 as a bona fide cellular substrate of NS3/4A that is required for HCV replication.

NS3/4A interacted with DDB1 in both mammalian overexpression system or HCV-infected cells. Site-directed mutagenesis indicated NS3/4A cleaved DDB1 after C378. The interaction between NS3/4A and DDB1 was not cleavage-dependent because NS3/4A could interact with the non-cleavable DDB1(C378R) mutant and the enzymatic-inactive NS3/4A(S139A) mutant interacted with DDB1 (Supplementary Fig. 5). Overexpression of DDB1 promoted HCV replication, whereas knockdown of DDB1 inhibited HCV viral RNA replication. These findings suggest that DDB1 plays an essential role in HCV replication.

Site-mutagenesis indicated that DDB1 was cleaved by NS3/4A after C378. Several experiments suggest that the cleavage of DDB1 is important for its role in HCV replication. Firstly, overexpression of all the non-cleavable DDB1(C378R), DDB1(S379N) and DDB1(G373A) mutants inhibited HCV replication; Second, reconstitution of the RNAi-knockdown cells with wild-type but not the non-cleavable mutant DDB1 restored HCV replication. These results suggest that both DDB1 itself and its cleavage are important for HCV replication. Interestingly, although the cleavage of DDB1 is important for HCV replication, the two cleaved fragments, either individually or in combination, had no marked effects on HCV replication in untransduced or DDB1-knockdown cells. These results suggest that the cleaved fragments do not contribute to HCV replication. The simplest explanation for these observations is that DDB1 functions at two or more steps in HCV replication. In an early event, HCV replication requires full-length DDB1. After that, cleavage of DDB1 itself or de-regulation of the Cul4-DDB1 E3 complex, but not the cleaved fragments, is required for a later step of HCV replication. However, the mechanism might be much more complicated and more studies are needed to elucidate them. Currently, we are also unable to exclude the possibility that mutations of DDB1 simply result in inactivation of its biological functions.

Since DDB1 is a component of the Cul4-DDB1 E3 ligase complex, we examined whether NS3/4A could be ubiquitinated following DDB1 overexpression. The results indicated that overexpression of DDB1 or DDB1(C378R) had no effects on NS3/4A ubiquitination and stability (Supplementary Fig. 9), excluding the possibility that the Cul4-DDB1 E3 ligase complex regulates NS3/4A ubiquitination and stability.

It should be pointed out, overexpression or knockdown of DDB1 could affect cell viability or proliferation. However, this starts to be observed only at 5–8 passages after its overexpression or knockdown. Therefore, we routinely used newly prepared cells for our experiments. We have used retrovirus-mediated gene transfer methods for transducing Huh7 cells with DDB1 or its RNAi plasmid. The transduced cells were selected with 4 μg/ml puromycin for three days and the positive cells were used for the experiments immediately. We found that the GADPH mRNA levels in DDB1 overexpression or RNAi expressing cells had no marked differences in comparison to control cells in real-time PCR experiments, indicating that the cells were comparable in terms of viability or proliferation during our experiments and the experimental results on the roles of DDB1 in HCV replication are reliable.

DDB1 is a multifunctional protein evolutionally conserved from fission yeast to humans (Tang and Chu, 2002). DDB1 was first identified in a complex involved in UV-mediated DNA damage response (Hwang et al., 1996). Mounting evidence has established DDB1 as a core subunit of the Cul4A and Cul4B-based ubiquitin ligase complex (Chen et al., 2001). In complex with Cul4, DDB1 has been proposed to either directly dock a substrate to the E3 machinery or indirectly recruit a substrate through a DCAF (DDB1 Cul4 Associated Factor), which usually contains WD40 repeats. Cul4-DDB1-DCAF forms as many as 90 E3 complexes that regulate diverse cellular pathways (Angers et al., 2006). Interestingly, it has been reported that the Cul4-DDB1 ubiquitin ligase machinery is a good hijack target for viruses. For example, it has been shown that the V protein of SV5 and the HBx protein of HBV can interact with DDB1 and hijack the E3 machinery for STAT1 degradation and virus replication (Andrejeva et al., 2002a; Andrejeva et al., 2002b; Didcock et al., 1999; Leupin et al., 2005), In addition, the HIV Vpr was also reported to interact with DDB1 and VprBP (a kind of DCAF) (Bergamaschi et al., 2009; Casey et al., 2010; Schrofelbauer et al., 2007; Sharova et al., 2008). Recently, DDB1 has been reported to be involved in infection of influenza virus, human immunodeficiency virus and cytomegalovirus (Bortz et al., 2011; Sharova et al., 2008; Trilling et al., 2011). However, how DDB1 affects the life cycles of these viruses is still unclear.

Structural studies indicate that DDB1 is a large multi-domain protein featuring an independent β-propeller domain (BPB) flexibly connected to a clam-shaped double-propeller fold(BPA–BPC) (Li et al., 2006). The BPA-BPC clam-shaped fold is responsible for recruitment of distinct DCAFs (Angers et al., 2006). Interestingly, both the V protein of SV5 and HBx interact with DDB1 at its clam-shaped fold junction, where C378 is located on a loop of the BPC region of this clam-shaped fold junction. It has been proposed that interaction of the V and HBx proteins with DDB1 may change the conformation of the BPA-BPC fold, and this may affect the recruitment of DCAFs and promote viral replication. In this context, it is possible that cleavage of DDB1 by NS3/4A may affect the formation of the Cul4A-DDB1-DCAF complex, a process required for HCV replication.

Since the Cul4A-DDB1-DCAF complex plays multiple roles in cells, the cleavage of DDB1 by NS3/4A may affect the conformation of this complex, leading to dis-regulation of the signaling cassettes involved. Recently, it has been shown that hepatocyte-specific deletion of DDB1 induces liver regeneration and tumorigenesis (Yamaji et al., 2010). In this context, it is possible that NS3/4A-mediated cleavage of DDB1 may contribute to hepatic carcinogenesis induced by HCV infection. Cul4A-DDB1-DCAF complex can regulate the histone methylation that control the epigenetic regulation (Higa et al., 2006; Pazhouhandeh et al., 2011), and it has been reported that virus infection may regulate this modification and contribute to development of carcinoma (Fernandez and Esteller, 2010; Flower et al., 2011; Paschos and Allday, 2010). Although many details regarding the involvement

of DDB1 in HCV replication and the significance of its cleavage by NS3/4A need to be further investigated, the identification of DDB1 as a cellular substrate critical for HCV replication provides new insights into the networks of HCV-host interaction. Furthermore, the finding that knockdown of DDB1 dramatically inhibited HCV replication and viral-particle production clearly justifies DDB1 as a potential target for drug development against HCV infection and related diseases.

Materials and methods

Reagents and antibodies

Mouse monoclonal antibodies against Flag, HA, and β-actin (Sigma), HCV-NS3 (Abcam), HCV-Core(Santa Cruz Biotechnology); rabbit monoclonal antibodies against the C-terminus of DDB1 (Epitomics), rabbit polyclonal antibodies against the N-terminus of DDB1 (Santa Cruz Biotechnology, Proteintech Group); horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and anti-rabbit IgG (Thermo); Alexa Fluor 555-conjugated anti-human IgG, Alexa Fluor 532-conjugated anti-mouse IgG; Hoechst 33258 (Invitrogen); and the NS3/4A inhibitor VX-950 (Selleck) were purchased from the indicated companies. Anti-E2 antibody was provided by Dr. Dennis Burton (The Scripps Research Institute, USA). Rabbit antiserum against HCV-NS3 was raised against NS3 recombinant protein. The Huh-7 and Huh-7 .5.1. cells were previously described (28).

Constructs

The pUC-JFH1 plasmid has been described previously (Zhong et al., 2005). The pCMV-DDB1 plasmid was purchased from Origene. Mammalian expression plasmids for HA- or Flag-tagged human DDB1, Cul4A and DDB2 were constructed by standard molecular biology techniques. DDB1 truncated mutants were constructed in the retroviral vector pMSCV that contains a puromycin selection marker (provided by Dr. Z. Huang, Wuhan University). NS3/4A was amplified by PCR from the pHCV-WHU plasmid (provided by Dr. C. Zheng, Wuhan University) and then cloned in frame with an N-terminal Flag or HA tag. The S139A mutant of NS3/4A, the C378R, S379N and G373A and RNAi off-target mutants of DDB1 were generated by site-directed mutagenesis. The pVSV-G and pGag-pol plasmids were provided by Dr. Z. Huang (Institut Pasteur de Shanghai).

Cells and transfection

The hepatic cell lines (Huh-7 and Huh-7 .5.1.) and 293 cells were maintained in complete Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 10 mM HEPES, 100 μg/ml penicillin, and 100 μg/ml streptomycin. The culture of Huh-7 Con1 subgenomic replicon needs an extra condition of 300 μg/ml G418. Transfection of 293 cells was carried out by the calcium phosphate precipitation method. Huh7 cells were transfected using lipofectamine 2000 (Invitrogen).

Protein purification and mass spectrometry analysis

The 293 cells (1×10^8) were transfected with an expression plasmid for Flag-NS3/4A or empty control vector. Eighteen hours post transfection, the transfected cells were lysed, and the lysates were immunoprecipitated with anti-Flag conjugated agarose beads. The anti-Flag associated proteins were eluted by Flag peptides and the eluted proteins were digested by trypsin in solution. The tryptic peptides were analyzed by HPLC-ESI/MS/MS using a Q-STAR Elite (Applied Biosystem) equipped with a nanospray ionization source using conditions as previously published (Chen et al., 2011).The tandem spectra were searched against National Center for Biotechnology Information reference database using the Mascot and Protein Pilot. Results were grouped by Scaffold and compared with control and other unrelated purifications.

Coimmunoprecipitation and immunoblot analysis

For transient transfection and coimmunoprecipitation experiments, 293 cells (1×10^7) were transfected for 24 h. The transfected cells were lysed in 1 mL of lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride). For each immunoprecipitation, 0.4 mL aliquot of lysate was incubated with 0.5 μg of the indicated antibody or control IgG and 30 μL of a 1:1 slurry of Protein G Sepharose (GE Healthcare) for 4 h. The Sepharose beads were washed three times with 1 mL of lysis buffer containing 500 mM NaCl. The precipitates were analyzed by standard immunoblotting procedures.

For endogenous coimmunoprecipitation experiments, Huh-7 cells (5×10^7) were mock-infected or infected with JFH-1 for the indicated times. The coimmunoprecipitation and immunoblot experiments were performed as described above.

HCV infection, replication and secretion

HCV culture was described previously (Zhong et al., 2005). HCV titers were determined with Huh-7 .5.1 cells by endpoint dilution and immunostaining as described previously (Zhong et al., 2005). HCV RNA levels were determined by quantitative reverse transcription-PCR (RT-qPCR) as described previously (Gastaminza et al., 2006). The RT-qPCR were performed on Bio-Rad CFX. Sequences of RT-qPCR primers are as following:

GAPDH
5′-GAAGGTGAAGGTCGGAGTC-3′ (sense)
5′-GAAGATGGTGATGGGATTTC-3′ (antisense);
JFH-1
5′-TCTGCGGAACCGGTGAGTA-3′ (sense)
5′-TCAGGCAGTACCACAAGGC-3′ (antisense)
Con1
5′-GCATATGACACCCGCTGTT-3′(sense)
5′-GTTTGACCCTTGCTGTTGA-3′(antisense)

Indirect immunofluorescent microscopy

Intracellular immunostaining was performed as described previously (Zhong et al., 2005). Briefly, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 . HCV E2 was stained with a human monoclonal anti-E2 antibody (C1) and followed with an Alexa Fluor 555-conjugated secondary antibody; HCV Core was stained with a mouse monoclonal anti-Core antibody and followed with an Alexa Fluor 532-conjugated secondary antibody. DNA was stained with Hoechst 33258.

HCVpp preparation and infection

HCV pseudotyped retroviral particles (HCVpp) were generated as previously described (Hsu et al., 2003). Briefly, 293 cells were co-transfected with the envelope-deficient HIV genome pNL4-3 .Luc.R-.E- and a plasmid expressing HCV glycoproteins. The medium was replaced with complete medium after 18 h. Supernatants were collected after 72 h and clarified by centrifugation and filtration. For infection experiments, HCVpp supernatants

were used to infect Huh-7 .5.1 cells (1×10^4) for 72 h. The infected cells were lysed with 20 μl of cell lysis reagent (Promega) and measured by luciferase reporter assays.

RNAi

Double-strand oligonucleotides corresponding to the target sequences were cloned into the pSuper.Retro RNAi plasmid (Oligoengine Inc.). The following sequences were targeted for human DDB1 cDNA: DDB1-RNAi #1, GTGACTTGATTGAGAGTTT; #2, GGATGAGTGCTGTGGAAAT.

Establishment of stable cell lines

The 293 cells were co-transfected with pSuper.Retro or pMSCV, pGag-pol and pVSV-G at a ratio of 3:3:1. The medium was replaced with complete medium after 18 h. Supernatants were collected after 72 h and clarified by centrifugation and filtration. Huh-7 cells were infected by the pseudotyped retroviral particles and selected by 4 μg/ml puromycin for 3 days.

Acknowledgment

We thank Drs. Dennis Burton, Congyi Zheng, Zan Huang and Zhong Huang for reagents. This work was supported by grants from the National Natural Science Foundation of China (30921001), and the Ministry of Science and Technology of China (2012CB910200, 2012ZX10002007).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2012.10.025.

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